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AUTHOR(S):

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Effect of an artificial silk elastin-like protein on the migration and collagen production
of mouse fibroblasts

Chisa Ozaki^{1,2}, Satoshi Somamoto², Shingo Kawabata², Yasuhiko Tabata¹

Short / Running Title: cell activities of an artificial SELP

¹ Department of Biomaterials, Institute for Frontier Medical Sciences, Kyoto University,
53 Kawara-cho Shogoin, Sakyo-ku Kyoto 606-8507, Japan

²Sanyo Chemical Industries Ltd.,
1-40, Goryo Ohara, Nishikyo-ku, Kyoto 615-8245, Japan

TEL +81-75-751-4121

FAX +81-75-751-4646

E-mail: yasuhiko@frontier.kyoto-u.ac.jp

Abstract

A silk elastin-like protein (SELP) is an artificial compound composing silk fibroin-like and elastin-like tandem repeats. The objective of this study is to evaluate the SELP effect on the migration, proliferation, and proteins production of L929 mouse fibroblasts. Upon culturing with different concentrations of SELP, the cells migration and their collagen production significantly enhanced in the SELP concentrations from 10^{-3} to 10 $\mu\text{g/ml}$. However, irrespective of the SELP concentration, no difference in the production of fibronectin, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor 1 α (SDF-1 α) was observed. When the migration of mouse peritoneal macrophages by SELP was evaluated, significant enhancement of macrophages migration was observed in any concentration. It is concluded that the SELP has a potential to promote the migration of fibroblasts and macrophages, and the fibroblast collagen production.

Keywords: artificial silk elastin-like protein, wound healing, cell migration

1. Introduction

Skin wounds disrupt the normal physiology of skin tissue, when the wound is created, the healing mechanism is initiated to re-establish skin continuity. Promotion of healing is often accompanied by the utilizing wound dressings [1, 2]. The dressing should give a favorable moist environment for wound healing, allow gas permeability, and protect the wound from the attack of bacteria. In addition, wound dressings need to be non-toxic and non-adherent [3-5]. Current strategies focus on the acceleration of wound repairing by systematically designed dressing materials [6]. Some researchers have experimentally and clinically demonstrated the materials which can accelerate the healing processes at molecular, cellular, and systemic levels [7-10].

Typical wound healing consists of three phases: inflammation, proliferation, and remodeling. At the inflammation phase, macrophages appear at the wound site. These cells, in addition to aggressively removing necrotic or foreign debris and phagocytizing bacteria, initiate two important aspects of healing process—angiogenesis and fibroplasia [11-16]. They are mediated by various proteins or cytokines released by activated macrophages. At the second proliferation phase, fibroblasts produce various components of extracellular matrix including collagen and fibronectin, and then generate granulation tissues [17]. At the remodeling phase, fibroblasts produce and remodel the extracellular matrix, and decrease in size of the underlying contractile connective tissue. In this connection, the fibroblasts migration into the wound, higher collagen production, and fibroblasts proliferation play a key role in the acceleration of wound healing process [18-21]. However, it should be noted that the fibroblast phenotype and collagen arrangement are also important factors to be considered for wound healing. The wound dressings to promote wound healing at the molecular level is of particular interest.

Silk elastin-like protein (SELP) is produced by recombinant DNA technology with relevant genes of silkworm fibroin and human elastin to generate the peptide repeats of silk fibroin (GAGAGS) and elastin-like (GVGVVP) units, respectively [22]. SELP shows biocompatibility and high elasticity of human elastin combined with the mechanical and tensile strength of silk fibroin in the molecular structure which is not present naturally in one molecule [23]. At concentrations of 4 wt% or higher, water-soluble SELP at room temperature can form a hydrogel at the body temperature [24]. Before application, SELP is in a liquid state, but can be solidified to form hydrogel at the body temperature. The area applied is automatically covered with the SELP hydrogel, and given in a moist wound healing condition without inflammation. This property of self-gelation is useful as the material of wound covering. SELP has a unique property to suppress cells adhesion without their apoptosis [25]. The biocompatibility of SELP is experimentally

confirmed by the intradermal injection to guinea pigs [23]. The medical and pharmaceutical applications of SELP to tissue engineering and drug delivery system have been investigated. [26-28].

This study is undertaken to obtain the fundamental knowledge of SELP properties for the cell behavior. As one of the representation fibroblast cell lines, L929 cells of mouse fibroblasts were incubated at different concentrations of SELP to evaluate the effect of SELP concentration on the migration, proliferation, and proteins production of fibroblasts. We examine the SELP effect on the migration of macrophages which is one of the key cells in the process of wound healing.

2. Materials and Methods

2.1. Materials

A silk elastin-like protein (SELP) is composed of four silk fibroin-like blocks, seven elastin-like blocks, and one modified elastin block containing a lysine (K) substitution (MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS [(GVGVVP)₄ GKGVP (GVGVVP)₃ (GAGAGS)₄]₁₂ (GVGVVP)₄ GKGVP (GVGVVP)₃ (GAGAGS)₂ GAGAMDPGRYQDLRSHHHHHH) [22]. The SELP was kindly supplied from Sanyo Chemical Co., Ltd., Kyoto, Japan.

2.2. L929 cells culture

A cell line of mouse fibroblasts (L929) was cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco Lifetechnologies Co., Carlsbad, CA) containing 10 vol% fetal bovine serum (FBS) (Gibco Lifetechnologies Co., Carlsbad, CA) and 1.0 wt% penicillin and streptomycin (Sigma-Aldrich Co., St. Louis, MO) or that containing 1.0 wt% penicillin and streptomycin (FBS-free DMEM) in 5% CO₂ and 95% air atmosphere at 37°C.

2.3. In vitro cell migration assay

In vitro cell migration as a wound healing assay was carried out according to the method described by Nakao et al. (2008). L929 cells were plated into each well of 6-well multi-well plates (Corning Inc., NY, USA) with 2 ml of DMEM at a concentration of 5×10^4 cells/well and cultured for 7 days to make cells confluent. Then, a portion of cells monolayer was scratched with a sterile 100 μ l plastic pipette tip to generate a linear wound as shown in Figure 1. The cellular debris was completely removed by washing with phosphate-buffered saline solution (pH 7.4, PBS). Then, DMEM containing 10^{-5} – 10^3 μ g/ml SELP (2 ml/well) was added to the well, followed by cell incubation for 0, 24, and 72 hr. The photographs of cells were taken on phase-contrast microscope (CKX41, Olympus Optical Co., Japan). The rate of the scratched area covered by migrated cells was assessed by Image J (National Institutes of Health, Bethesda, MD). The migration ratio was quantitated in three random fields for each experimental sample. The distance of cells migrated after incubation at the SELP concentration of 0 μ g/ml is defined as 1.0 to calculate the migration ratio for samples. To exclude the effect of cell proliferation, the similar experiment was performed at a concentration of 5 μ g/ml mitomycin C (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.4. Cell proliferation assay

Cell proliferation was evaluated according to the method reported previously [29]. L929 cells were plated into each well of 24-well multi-well plates (Corning Inc., NY, USA) with 250 μ l of DMEM at a concentration of 2×10^5 cells/well, followed by 6 hr culture to allow cells to attach. Then, 250 μ l of DMEM containing $2 \times 10^{-5} - 2 \times 10^3$ μ g/ml SELP or 5 μ g/ml basic fibroblast growth factor (bFGF) was added to the well. After culturing for 18 and 66 hr, 40 μ l of a tetrazolium-used colorimetric MTT assay cell-counting reagent (Nakalai tesque Co., Japan) was added into each well. After 2 hr of incubation, the absorbance of each well at 450nm was measured using a UV-microplate reader (VERSAmax, Molecular Devices Inc, USA). The number of cells was calculated with a standard curve prepared by using the known numbers of cells. The experiment was performed independently 3 times for each sample unless otherwise mentioned.

2.5. Analysis of collagen production

Trypsinized L929 cells were plated into each well of 6-well multi-well plates with 1.0 ml of DMEM at a concentration of 2×10^5 cells/well, followed by 6 hr culture to allow cells to attach. Then, 1.0 ml of DMEM containing $2 \times 10^{-5} - 2 \times 10^3$ μ g/ml SELP was added to the well, and cultured for 3, 5, 7, 9, and 11 days. Then, the total amount of collagen produced was measured with a collagen stain kit based on Sirius red / Fast green staining (collagen research center Ltd., Japan) according to the manufacturer's protocol.

2.6. Enzyme-linked immunosorbent assay (ELISA) for fibronectin, growth factors, and chemokine

Trypsinized L929 cells were plated into each well of 24-well multi-well plates with 250 μ l of DMEM at a concentration of 2×10^5 cells/well, followed by 6 hr culture to allow cells to attach. Then 250 μ l of DMEM containing $2 \times 10^{-5} - 2 \times 10^3$ μ g/ml SELP or 10 μ g/ml transforming growth factor- β 3 (TGF- β 3) was added to the well. After culturing for 3 days, the total amount of fibronectin, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor 1 α (SDF-1 α) were measured with a VEGF ELISA kit (R&D systems Inc., USA), bFGF ELISA kit (ray biotech Inc., USA), SDS-1 α ELISA kit (R&D systems Inc., USA) and fibronectin ELISA kit (Biomedical Technologies Inc., USA) according to the manufacturer's protocol.

2.7. Macrophage migration assay

Peritoneal macrophages were obtained from of C57BL/6 mice (6–9 weeks, SHIMIZU Laboratory Supplies Co., Kyoto, Japan) by the conventional peritoneal lavage method

[30]. Prior to 4 days, mice were intraperitoneally injected by 4 w/v % thioglycolate medium (2 ml). The mice were sacrificed by the extravasation, and then 5 ml of cold PBS was injected into the peritoneal cavity. The peritoneal cavity was massaged for 3 min. And, the peritoneal fluid was collected by syringe aspiration. The cell suspension obtained was centrifuged at $1000 \times g$ for 5 min at 4°C and adjusted to 5×10^6 cells/ml with RPMI-1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 1.0 wt% penicillin-streptomycin.

The cell migration assay with a boyden chamber was performed for peritoneal macrophages by using transwell chambers with a 8- μm pore-sized membrane (Corning Inc., NY, USA). The chambers were inserted into each well 24-well multi-well culture plates containing 0 and $10^{-5} - 10^3 \mu\text{g/ml}$ of SELP or 10^{-8} M N-Formyl-Met-Leu-Phe (FMLP) in FCS-free RPMI-1640. Macrophages (5×10^5) were placed into the upper portion of boyden chamber. The non-migrating cells were removed with a cotton swab 3 hr later, and cells were fixed in methanol for 15 min and stained with crystal violet. The photographs of cells on transwell membranes were taken on the phase-contrast microscope. The photographs of cells migration were viewed to assess the number of cells migrated across membrane. The viewing was performed independently 3 times for each experimental sample. The number of cells migrated after incubation in the absence of SELP is defined as 1.0 to evaluate the migration ration of samples.

2.10. Statistical analysis

Statistical analyses were performed by using Student's t-test. The P-value less than 0.05 were considered to be significant. Data were expressed as the mean \pm the standard deviation.

3. Results

3.1. Migration and proliferation of L929 cells cultured with SELP

Figures 1A – 1D show the effect of SELP concentration on the migration of L929 cells. The migration of L929 cells enhanced at the SELP concentrations of 10^{-1} and $10 \mu\text{g/ml}$ to a significant extent compared with that at $0 \mu\text{g/ml}$, 24 hr after addition of SELP. The similar result was obtained at 72 hr later, but the influence of SELP concentration on the cell migration was different from that 24 hr later. The picture of cell migration also showed the similar, effect of SELP concentration. The similar experiment was performed for cells which had been treated with mitomycin C, which inhibits the DNA synthesis and consequently cell proliferation. The same effect of SELP concentration on the cells migration was observed (Figure 1E).

Figure 2 shows the effect of SELP concentration on the proliferation of L929 cells. bFGF showed an acceleration effect on the cell proliferation. However, the cells proliferation was not enhanced by the SELP, irrespective of the concentration.

3.2. SELP induced collagen production in fibroblast cells

Figure 3 shows the effect of SELP concentration on the collagen production of L929 cells after incubation at different concentrations of SELP. When culture at the SELP concentrations of 10^{-5} , 10^{-3} , 10^{-1} , and $10 \mu\text{g/ml}$, the collagen production enhanced significantly 7 and 9 days after incubation, compared with that at different concentrations.

3.3. Proteins production of L929 cells cultured with SELP

Figure 4 shows the effect of SELP concentration on the production of key proteins for wound healing process. No difference in the production of bFGF, VEGF and SDF-1 α was observed, irrespective of the concentration of SELP. The level of fibronectin production was not changed by the addition of SELP at any concentration. The addition of transforming growth factor- β 3 (TGF- β 3) as a positive control enhanced the production of fibronectin.

3.4. Macrophage migration of SELP

Figure 5 shows the effect of SELP concentration on the migration of macrophages. When culture at the SELP concentrations of 10^{-3} , 10^{-1} , and $10 \mu\text{g/ml}$ or N-Formyl-Met-Leu-Phe (FMLP) as a positive control, macrophages migration enhanced to a significant extent compared with that at different SELP concentrations.

4. Discussion

We have experimentally confirmed that SELP accelerated the wound healing of a full-thickness defect with a diabetic mouse and a guinea pig models (under submission). In the animal experiments, the promotion of granulation tissue formation was observed. The objective of this study is to evaluate the cellular mechanism of granulation promotion by in vitro cell culture experiments. Considering the type of cells working at the inflammation process, fibroblasts and macrophages were selected. Their migration and proliferation was evaluated. It is well known that the collagen production is one of the key events in the formation of granulation tissues [31]. In addition, growth factors and chemokines of bFGF or VEGF and SDF-1 α also play an important role in the wound healing [32]. The present study indicates that the SELP enhanced the migration of L929 cells and their collagen production at a certain range of concentration. However, the cell proliferation and the production of bFGF, VEGF, SDF-1 α , and fibronectin were not changed in the presence of SELP.

There are a lot of reports about response of cell to elastin and silk fibroin [33-45]. Unlike in all the others, we could show that SELP promote the wound healing by using the in vitro cell culture. SELP already has suitable properties for the wound dressing (for example, temperature-responsive gelation, high elasticity, and biological compatibility). In addition, we could enhance the value of SELP as the material of wound dressing, through this paper.

Macrophages represent one of the most abundant inflammatory cell types during all stages of wound healing [46]. At the first phase of inflammation, macrophages are key cells, which can control the subsequent processes [47, 48]. Their primary function upon recruitment from blood into the damaged area is to clear cellular debris and necrotic tissue. And macrophages sense, and combat invading pathogens. A reduced macrophages recruitment causes retarded wound healing [49]. In addition, macrophages synthesize numerous growth factors contributing to physiological and pathological tissue growth [50, 51]. In this study, the macrophages migration was evaluated. It is apparent that the macrophages migration was significantly enhanced by the SELP (Figure 5).

Elastin molecule consists of hydrophobic domains with abundant Gly, Val, Ala, and Pro residues which often occur in repeats of several amino acids, like Gly-Val-Gly-Val-Pro (GVGVP), Gly-Val-Pro-Gly-Val (GVPGV) and Gly-Val-Gly-Val-Ala-Pro (GVGVAP). Elastin sequences interact with a variety of cell types to modulate their behavior. Insoluble elastin, for example, has been shown to mediate the cell adhesion of monocytes, fibroblasts, and tumor cells. Elastin digests and elastin derived peptides have also been shown to be chemoattractants for monocytes and fibroblasts [33-36].

Elastin degradation products have also been shown to act on calcium ion channels in monocytes, fibroblasts, and smooth muscle cells [37], and inhibit platelet aggregation [38].

Several reports on the effect of specific elastin sequence on cell behavior have been reported [34, 35, 39]. Both the monomer and the polymer of VPGVG, which is almost similar sequences with SELP elastin block, showed no significant effect on the proliferation of skin fibroblasts, while both the monomer and the polymer of VGVAPG enhanced the cells proliferation and down-regulated the elastin or collagen mRNA levels [39]. The research supports this report that SELP had no positive effect on the cell proliferation.

It have also been reported that the monomer and the polymer of the hexapeptide, GVGVP were chemotactic for bovine ligamentum fibroblasts, while the monomer and the polymer of the pentapeptide, GVGVP which is contained in SELP, were not chemotactic [35]. However, SELP enhanced the migration of L929 cells and macrophages (Figures 1 and 5). Ligamentum nuchae fibroblasts are not migrated by the hexapeptide [34]. It suggests the possibility that different portions of elastin molecules would have an ability to recruit different types of cells. The signaling pathway of elastin peptides, including GVGVP and GVGVP, have been reported. Depending on the cell type, various signaling modules are triggered, converging to the activation of the extracellular signal-regulated kinase 1/2 (ERK1/2), which appear as crucial actors in elastin peptide signaling [40, 41]. ERK1/2 is the main subfamily of mitogen-activated protein kinases (MAPKs), which are linked to the cellular migration in a number of systems [42]. It is probable that the ERK1/2 pathway is involved with promoted cell migration by SELP. However, the detailed mechanisms of events remain unknown. Further study is needed to reveal the signaling pathway triggered by SELP.

Silk fibroin is a structural protein, and is considered to be a suitable material for skeletal tissue engineering because of its good oxygen and water-vapor permeabilities and its in vivo minimal inflammatory reaction [52]. In addition, researchers have investigated the biological effects of silk fibroin as a matrix, and concluded that silk fibroin has positive effects on cell adhesion, viability, growth and differentiated functions [43-45]. In this study, SELP used as a soluble agent added into the culture medium, and not as a matrix. Therefore, the function of SELP evaluated in this study cannot compare directly with that previously reported.

Silk fibroin-like blocks of SELP can be attributed to the formation of an extended crystalline β -sheet structure that is composed of recurrent sequences of Gly, Ala, and Ser amino acids, which in most cases begin with repeats of the GAGAGS hexapeptide [53, 54]. In addition, GAGAGS hexapeptide have no discernible impact on cell behavior.

Silk fibroin-like blocks of SELP reflect a promising material for biomedical applications, due to its biocompatibility, biodegradability and mechanical properties.

The data of SELP-induced cells activation are not always good enough to explain the in vivo effect of SELP on the promoted granulation formation. This may be explained by the difference in the state of SELP. In the case of in vivo application, the SELP concentration is high enough to set a hydrogel automatically. The hydrogel formation covers the skin defect, which may affect the acceleration of granulation formation. It is possible that the SELP hydrogels can be degraded in the body to release out the water-soluble fragments of SELP. We confirmed that SELP hydrogel, which contained 20 wt% of SELP and 80 wt% of moisture, was degraded in PBS, and release 2.3 ± 0.5 % at 0 hr, and 16.2 ± 0.5 % of SELP at 72 hr. Taken together, it is highly conceivable that in vivo the SELP released out of hydrogels acts on the fibroblasts or macrophages, resulting in the enhanced granulation. Considering the biological functions of SELP in the body, it is practically important to evaluate the effect on the cell behavior in both the hydrogel solid and water-soluble forms. This study focusses on the biological effects of latter soluble form. It is no doubt that the cellular effect of SELP hydrogel should be evaluated in future. In addition, further investigation of other mechanisms on the promoted granulation should be proceeded.

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Figure legends

Figure 1: Effect of SELP concentration on the migration of L929 cells. Migration was evaluated for 24 (A, B), and 72 hr (C, D) after incubation with SELP. And migration was also evaluated for 24 hr with the addition of mitomycin C (E). The migration ratio of cells cultured in the absence of SELP is defined as 1.0 to evaluate the migration ratio of samples. The scale bar is 300 μ m. *, †, $p < 0.05$; significant between the two groups.

Figure 2: Effect of SELP concentration on the proliferation of L929 cells. Cells were incubated with different concentrations of SELP, and 5 μ g/ml bFGF. The proliferation of cells cultured in the absence of SELP is defined as 1.0. *, $p < 0.05$; significant between the two groups.

Figure 3: Effect of SELP concentration on the collagen production of L929 cells 3 (□), 5 (▤), 7 (▥), 9 (▦), and 11 days (▧) after incubation with different concentrations of SELP. Cells were incubated with different concentrations of SELP. The collagen production of cells cultured in the absence of SELP is defined as 1.0. *, $p < 0.05$; significant between the two groups.

Figure 4: Effect of SELP concentration on the proteins production of L929 cells: bFGF (A), VEGF (B), SDF-1 α (C), and fibronectin (D). Cells were incubated with different concentrations of SELP, and 10 μ g/ml TGF- β 3. The proteins production of cells cultured in the absence of SELP is defined as 1.0. *, $p < 0.05$; significant between the two groups.

Figure 5: Effect of SELP concentration on the migration of macrophages. Migration was evaluated 6 hr after incubation with SELP and 10⁻⁸ M FMLP. The migration number of cells cultured in the absence of SELP is defined as 1.0 to evaluate the migration ratio of samples. *, †, ‡, † ‡, $p < 0.05$; significant between the two groups.

Fig.1

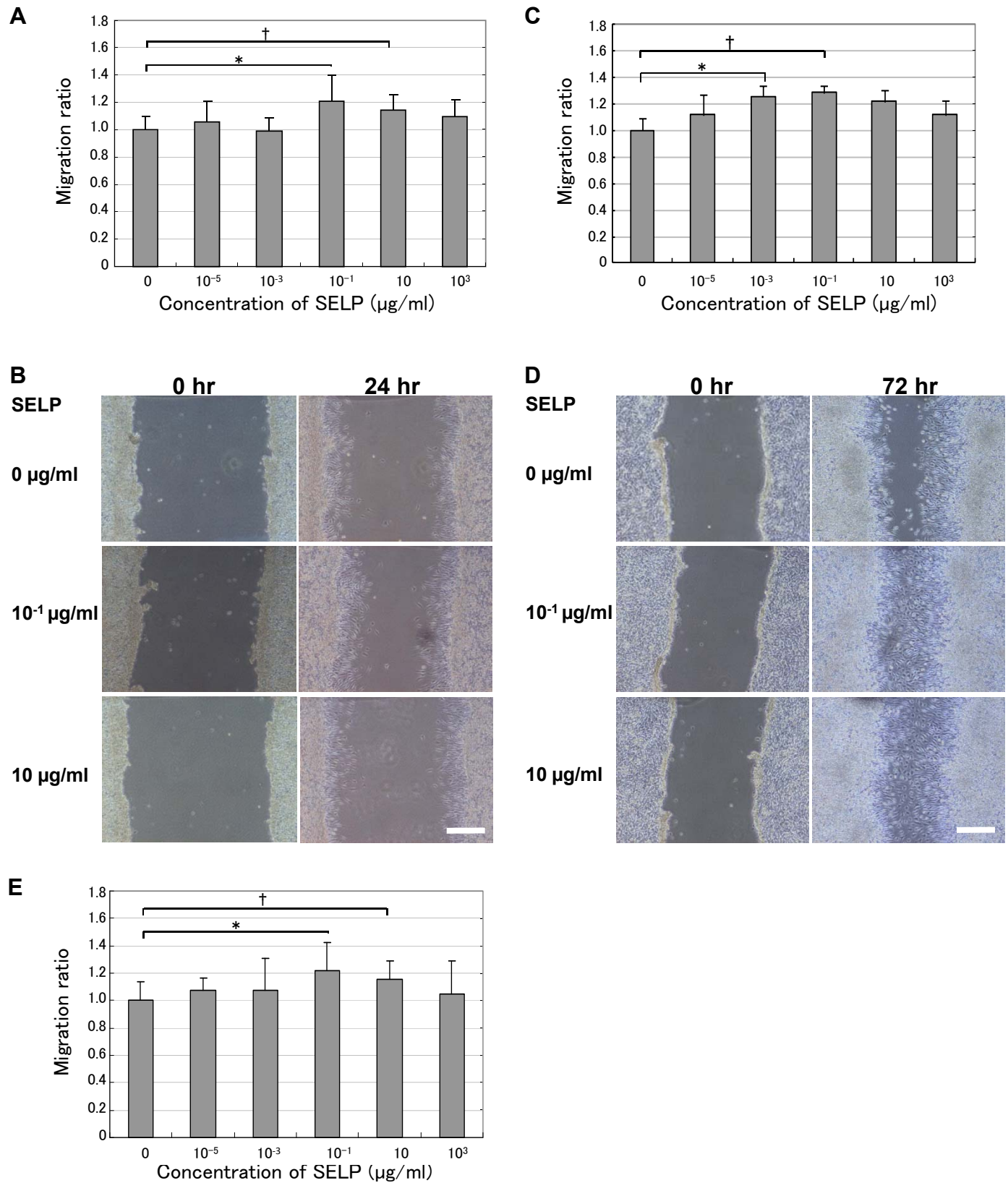


Fig.2

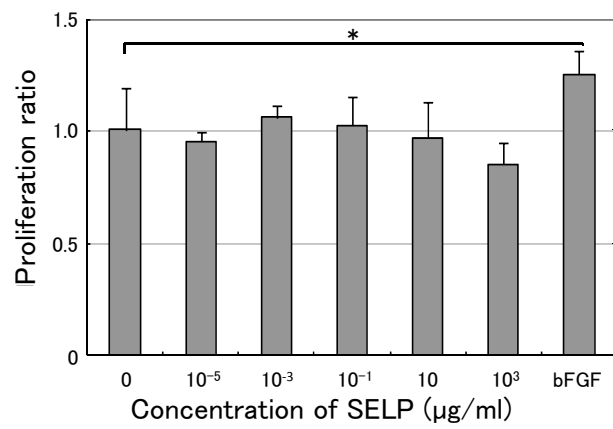


Fig.3

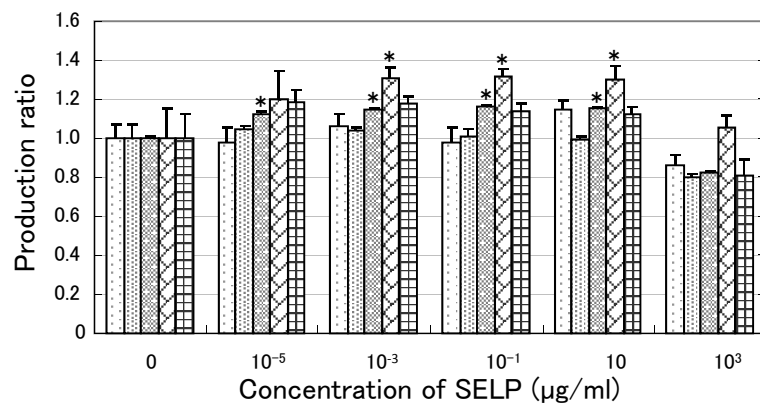


Fig.4

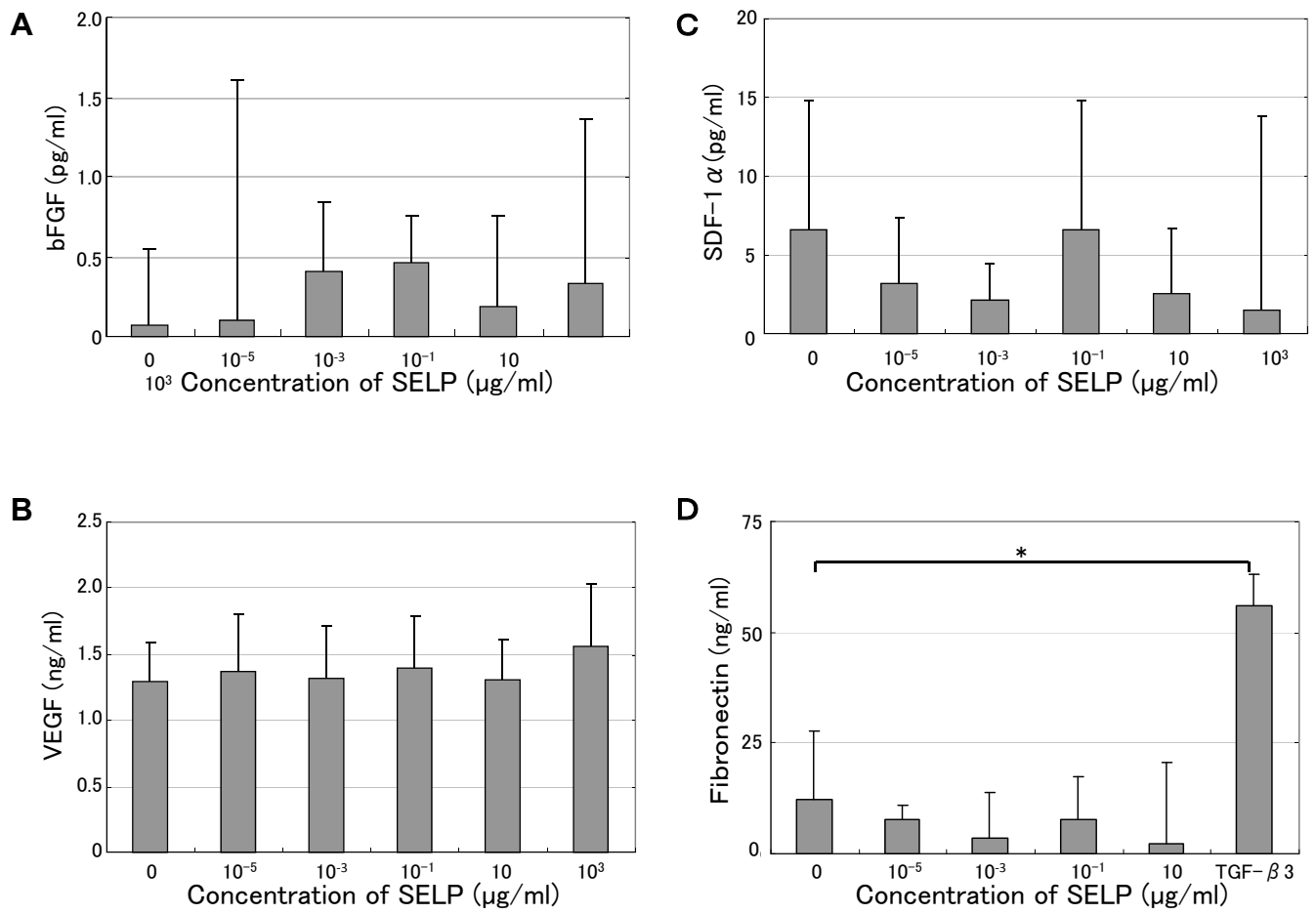


Fig.5

